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Inactivation of the Corpora Allata in the Final Instar of the Tobacco Hornworm, *Manduca sexta*, Requires Integrity of Certain Neural Pathways from the Brain

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Abstract. Neither the implantation of active CA nor treatment with O-ethyl,S-phenylphosphoramidothiolate (EPPAT), a potent inhibitor of the juvenile hormone esterase (JHE), prevented metamorphosis of final instar tobacco hornworms. However, a combination of the two treatments often blocked metamorphosis and caused the formation of supernumerary larvae or larval-pupal intermediates. So also, in conjunction with EPPAT treatment, unilateral severance of the medial nerve from the brain to the corpus cardiacum-corpus allatum complex often resulted in abnormal supernumerary or intermediate larval forms. Thus, clearance of JH from mature hornworm larvae prior to metamorphosis appears normally to depend on two mechanisms: (1) cessation of JH production by inhibition of the CA *via* the innervation of these glands, and (2) destruction of previously secreted existing JH *via* production of JHE. In the present experiments, each of these mechanisms appeared fully able to clear JH sufficiently to permit normal metamorphosis, because only simultaneous interruption of both mechanisms led to formation of supernumerary larvae. Acting in concert as they do late in larval life, these two mechanisms ensure the timely and thorough clearance of JH in preparation for metamorphosis.

Introduction

Juvenile hormone (JH) is indispensable for maintenance of the larval condition in Lepidoptera as it is in many other orders of insects. Conversely, metamorphosis normally presupposes the virtual absence of JH and can regularly be derailed by the timely application of JH or JH analogs to mature larvae. Thus the transition from the larva's feeding life-style to the metamorphic sequence entails elimination of JH. This prerequisite could potentially be satisfied either by curtailing production of JH or by augmenting its destruction. In fact, persuasive evidence supports regulation by both pathways. Thus, production of JH by the corpora allata (CA) declines late in larval life (for review, see Feyereisen, 1985); simultaneously, breakdown of JH to inactive metabolites is enhanced by the increased production of enzymes inactivating JH such as the so-called "juvenile hormone esterase" (JHE) (for review, see Hammock, 1985).

In the present communication, we focus on regulation of JH clearance in the final instar of the tobacco hornworm *Manduca sexta*. Prior studies of this species have documented a rapid increase in the activity of JHE during the final instar (Vince and Gilbert, 1977; Sparks *et al.*, 1983). The importance of this increase has been supported by the demonstration that administration of specific JHE inhibitors can delay metamorphosis by prolonging the final instar just as can be accomplished by administering JH or its active analogs (Sparks *et al.*, 1983). Moreover, studies on hornworm CA have shown that their activity declines during the final larval instar (Bhaskaran *et al.*, 1980). JH bioassays have directed attention to multiple avenues of CA regulation, including deployment of humoral and neural inhibitors as well as withdrawal of humoral and neural stimulators (Bhaskaran and Jones, 1980; Bhaskaran *et al.*, 1980; Bhaskaran, 1981). However, neither interruption of JH breakdown nor interference with CA regulation has successfully pro-

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Abbreviations: JH = juvenile hormone; JHA = juvenile hormone analog; JHE = juvenile hormone esterase; br-cc-ca = brain-corpora cardiaca-corpora allata complexes; CA = corpora allata; EPPAT = O-ethyl,S-phenylphosphoramidothiolate; LD = long day; SD = short day; *bl* = black larva strain of the tobacco hornworm.

voked supernumerary larval molts or done more than delay the normal pupation of final instar hornworms. Thus, the normal regulatory mechanisms that are critical for JH clearance in mature hornworm larvae remain uncertain. In the experiments described here, we document the key role played by JHE, as well as that of a brain-centered neural inhibition of JH secretion by the CA.

Materials and Methods

Hornworms were reared as previously described (Safranek and Williams, 1980) under a long-day (LD, 17L: 7D) or a short-day (SD, 12L:12D) photoperiod at 25°C. The first day of each instar was termed Day 1. Bioassays of brain-corpora cardiaca-corpora allata complexes (br-cc-ca) were performed in *black* (*bl*) hornworm larvae during the first half of the photophase as previously described (Safranek and Riddiford, 1975). Implantation of these complexes occurred 1–6 h prior to the onset of head capsule slipping in host larvae, and hosts were scored 48 h later after completion of the molt. The scoring system was derived from that previously described (Truman *et al.*, 1973) and is summarized here under Results.

Denervation of the CA was accomplished in CO_2- anesthetized larvae under Ringer's solution (Safranek and Williams, 1980). A small, three-sided flap of integument was raised near the center of the head capsule, thereby exposing the brain in the center of the field. With forceps placed behind the brain, the latter was delicately tipped forward—a maneuver that permitted adequate exposure of the two small nerves from one hemisphere of the brain to the ipsilateral corpus cardiacum-corpus allatum complex. After both nerves had been inspected, the one to be transected was securely grasped with forceps and carefully severed with scissors. The integumentary flap was replaced and the wound sealed by melted wax. In sham-operated preparations, both nerves were identified on one side but not manipulated. In all experiments, individuals were carefully examined after completion of the molt at the end of the fifth instar for JH-dependent morphological abnormalities of the types previously described (Truman *et al.*, 1974).

The juvenile hormone esterase inhibitor O-ethyl,S-phenylphosphoramidothiolate (EPPAT) was a gift of Drs. T. C. Sparks and B. D. Hammock. It was dissolved in ethanol for topical administration to the dorsal thorax or anterior abdomen using a Hamilton syringe and a repeating dispenser.

Results

Larval hornworm CA are inactivated prior to metamorphosis

To document inactivation of hornworm CA prior to metamorphosis, br-cc-ca complexes were dissected from SD fifth-instar hornworms and implanted for bioassay directly into fourth-instar *black* hornworm larvae as described under Materials and Methods. All operations were performed on *bl* larvae during the first half of the photophase so that the implanted complexes were present shortly before the onset of head capsule slipping by the *bl* larvae, at which stage the *bl* fifth-instar pigmentation can be modified by exposure to JH. The *bl* assay is useful when comparing the activity of br-cc-ca complexes producing amounts of hormone less than that required to generate the assay's maximum score of +3. As summarized in Figure 1, the complexes of larvae weighing less than 8 g on the morning of assay were nearly always active, whereas those from larvae weighing more than 8 g at that stage of the photocycle were nearly always inactive. Larvae weighing over 8 g during the first half of the photophase usually initiated metamorphosis (signaled by dorsal vessel exposure and onset of the wandering behavior) during the next scotophase; smaller larvae delayed metamorphosis for one or more days. Thus, in these experiments, complexes actively secreting JH were

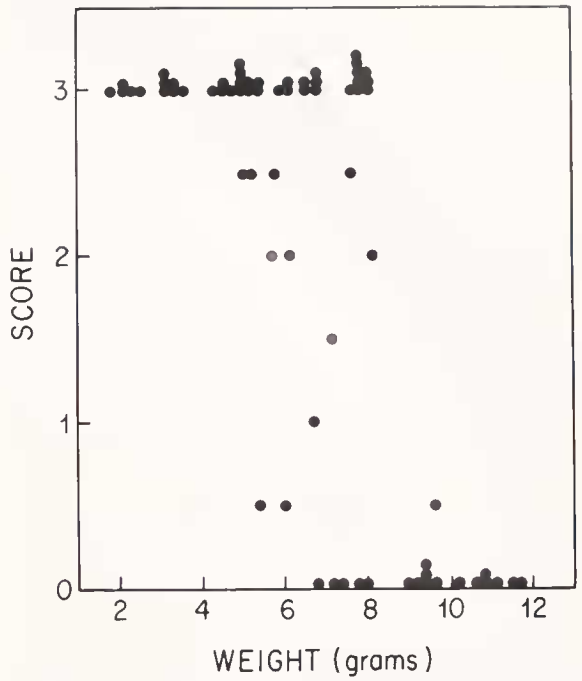

Figure 1. Activity of fifth-instar larval corpora allata in the *black* larval assay as a function of fifth-instar larval weight. Brain-cc-ca complexes from feeding fifth-instar larvae of various weights were singly implanted for assay into pharate fifth *black* hornworm larvae. These assay larvae were scored after completion of the molt to the fifth instar using the following scoring system: 0 = fully black pigmentation; 0.5 = grey head or thorax; 1.0 = green head or thorax; 1.5 = green head or thorax with faint green pigmentation apparent in the abdomen; 2.0 = grey-green abdomen; 2.5 = excessive melanization restricted to cuticular creases across the abdomen; and 3.0 = normal pigmentation. Each point represents assay of one brain-cc-ca complex.

regularly identified from larvae that were at least 36 h before the onset of the wandering period, whereas inactive complexes were found when donor larvae would have initiated wandering in less than 12 h. Thus, inactivation of the CA occurs from 12 to 36 h before the visible onset of the wandering period.

Denervated active CA block metamorphosis only in conjunction with EPPAT administration

A series of experiments were designed to evaluate the developmental responses to implantation of denervated CA in the presence or absence of EPPAT. For these studies we selected Day 2 LD fifths weighing 3.3–5.0 g, because the above data suggested that all larvae of this stage would have CA actively secreting JH. One group received implants of single CA from larvae of a similar weight and stage. A second group received application of 20 nmol of EPPAT (2 μl) every 8 h, beginning late on Day 2 and continuing until dorsal vessel exposure signaled the onset of metamorphosis. This dose and schedule of administration had been shown in preliminary experiments neither to delay the onset of metamorphosis by intact fifth-instar hornworms nor to give rise to morphological aberrations in the resulting pupae. A third group received implantation of a single CA on Day 2 followed by 20 nmol of EPPAT (2 μl) every 8 h beginning on Day 3 and continuing until the appearance of dorsal vessel exposure or of head capsule slipping. Three control groups received either a sham-operation or topical ethanol application (2 μl) every 8 h or a combination of sham-operation and topical EPPAT application (20 nmol EPPAT in 2 μl ethanol every 8 h). All larvae were restored to diet after initiation of treatment.

Results of these experiments are summarized in Table I. Larvae that received both implantation of isolated CA as well as topical application of EPPAT were the only group substantially different from the controls. Of the 23 larvae in this group, 12 formed larval-pupal intermediates similar to those seen after JH treatment of late fifth-instar larvae. Among these individuals, head capsule slipping occurred on average at Day 10 of the instar. Even the 11 larvae in this group that did not form intermediates appeared, nevertheless, to have been affected by the treatment: wandering was typically delayed until Day 10 of the instar and only 7 of the 11 pupated normally; 3 others died prior to pupation; and 1 displayed multiple abnormalities but no larval features. These findings were in contrast to the development of the control groups and of the groups in which either implantation of denervated CA or topical EPPAT application were singly employed: all groups initiated metamorphosis with, at most, slight average delays, and all individuals survived to form normal pupae.

Table I

Effects of treatment with EPPAT, denervated CA, or both

Treatment	Number	Days to wander or molt*	% Larval-pupal intermediate#
Sham operation	9	7/0	0
Sham (ethanol) application	19	6/0	0
Implantation of active CA	9	7/0	0
EPPAT application	19	6/0	0
Sham operation plus EPPAT application	20	8/0	0
CA implantation plus EPPAT application	23	10/10	52

* Mean day of fifth instar on which initiation of the wandering period or of head capsule apolysis was first noted. Treatment was initiated on LD fifth-instar larvae weighing 3.3–5.0 g late on Day 2.

Intermediates ranged from sixth-instar larvae of nearly normal appearance to largely pupal forms with retained prolegs and mandibles. The remainder exhibited patches of larval and pupal cuticle distributed in patterns characteristic of fifth-instar larvae treated with JH analogs (Truman *et al.*, 1974). Few supernumerary larvae or intermediates were able successfully to complete ecdysis at the end of the fifth instar, nor did any feed substantially or undertake further development before death.

We also attempted to abort the normal metamorphosis of fifth-instar larvae similar to those above (LD, Day 2, 3.5–5.0 g) by implanting individual loose CA from fourth-instar larvae (n = 22), or Day 1 fifth-instar larvae (n = 13), or adult females (n = 7). CA from all these stages are highly active in the *black* larval bioassay or in a pupal bioassay (Safranek and Williams, 1987; unpub. results). In no instance did the implanted CA prevent pupation or provoke retention of larval features.

Another experiment used older LD fifth instars approximately 30 h prior to the onset of the wandering period. Implantation of a single CA from a Day 1 fifth-instar (n = 13) or treatment with EPPAT (100 nmol every 8 h) (n = 9) did not prevent normal pupation. By contrast, combination of the two treatments once again led to the occurrence of sixth-instar larvae or larval-pupal intermediates in 5 of 8 larvae. Thus, as in the previous experiments, neither implantation of CA nor administration of EPPAT deployed individually substantially altered either the time course or character of metamorphosis: only combination of the two treatments regularly prevented normal pupation and led to retention of larval characteristics.

Not all EPPAT-treated preparations containing denervated CA formed larval-pupal intermediates or supernumerary larvae. Though we are uncertain of the reason for this, we suspect that the dosage of EPPAT used here may have been inadequate to ensure a response by all larvae. Our limited supply of EPPAT prevented extensive exam-

Table II

Effects of in situ *denervation of the CA coupled with EPPAT application*

Operation	Number of larvae#	% Larval-pupal intermediates*
Sham	27	0
Sever a lateral nerve to an active CA	19	11
Sever a medial nerve to an active CA	25	64

The numbers represented include only those surviving to molt. Four sham-operated preparations died postoperatively, as did three preparations with a severed lateral nerve, and five with a severed medial nerve.

* All individuals not forming larval-pupal intermediates formed pupae that were normal except for minor aberrations about the head believed secondary to the surgery. Larval-pupal intermediates demonstrated the same range of forms described in Table I. The heads of all preparations forming largely larval intermediates were dissected after the molt and in every case the persistent section of the originally operated nerve was confirmed.

ination of the response to higher EPPAT dosages. Moreover, we wished to avoid the use of EPPAT at higher dosages that cause developmental delays even in intact larvae without denervated CA (Sparks *et al.*, 1983; pers. obs.). Our limited experience with higher EPPAT dosages up to 200 nmol every 8 h suggests that more aggressive EPPAT-treatment to larvae with loose or denervated CA might well result in an even higher percentage of larval-pupal intermediates than described in the present experiments.

Denervation of CA in situ *can block metamorphosis in conjunction with EPPAT administration*

Table II summarizes a final series of experiments on fifth-instar hornworms; here we succeeded in unilaterally denervating active CA without otherwise disturbing them. In these experiments we used mature SD fifth instars 24–36 h prior to the outset of the wandering period. Larvae at this stage could be removed from diet postoperatively without the significant developmental delay routinely encountered after operation and starvation of younger larvae. Either the medial or the lateral nerve from the brain to the corpus cardiacum of one side was transected as described under Materials and Methods. After the operation, all individuals were removed from diet and received an initial dose of 120 nmol of EPPAT followed by 50 nmol every 8 h. As shown in Table II, transection of a medial nerve to a CA resulted in the formation of a larval-pupal intermediate by the majority of larvae.

Discussion

Denervation of the CA figured critically in the aborted metamorphosis of the larval-pupal intermediates generated in the present experiments. Either implantation of active CA or denervation of *in situ* CA often generated supernumerary larvae and larval-pupal intermediates when these maneuvers were deployed along with administration of the potent JHE inhibitor EPPAT. These aberrant forms were indistinguishable from the supernumerary larvae and larval-pupal intermediates seen after administration of large doses of JHA to mature feeding fifth-instar larvae (Truman *et al.*, 1974). We attribute the present findings to the inability of the brain to inactivate the denervated CA: the latter's continued secretion of JH could not be countered by the EPPAT-inactivated JHE. Hence, the secreted JH remained active and caused retention of larval features.

The present experiments document inactivation of hornworm CA late in the feeding portion of the final instar. Suppression of JH synthesis by the CA through the neural circuitry to those glands has been suggested in earlier work on the hornworm (Bhaskaran *et al.*, 1980) as well as in other species (for review, see Feyereisen, 1985). The anatomical basis for neural inhibition of the CA by the brain has also been documented: the hornworm CA demonstrate a complex innervation arising from the brain, including axons from multiple neurosecretory cells whose cell bodies lie in several regions of the brain (Nijhout, 1975; Carrow *et al.*, 1984). Most of the documented neurosecretory innervation of the CA from the brain is *via* the medial nerve (termed NCC I–II in the work of Nijhout, 1975) whose section in the present experiments coupled with EPPAT treatment often resulted in the production of larval-pupal intermediates.

Implantation of active CA can alone effectively prevent the pupation of allatectomized fourth-instar larvae and starved immature fifth-instar larvae (Bhaskaran and Jones, 1980; Bhaskaran *et al.*, 1980). The efficacy of implanted CA in these cases contrasts with our inability to block the pupation of mature, feeding fifth-instar larvae merely by implantation of active CA. In the present experiments denervation of the CA was of morphogenetic consequence only when deployed concurrently with the JHE inhibitor EPPAT. This is presumably explained by the high levels of JHE activity found in feeding fifth-instar larvae, levels manifestly sufficient to inactivate JH produced by denervated CA of every stage examined.

These experiments highlight the important role of JHE in the elimination of circulating JH prior to metamorphosis. Nevertheless, production of JHE does not seem to be required for clearance of JH sufficient to permit metamorphosis. For example, while treatment of fifth-instar larvae with EPPAT in dosages larger than

those deployed here can slightly delay the onset of metamorphosis (Sparks *et al.*, 1983; pers. obs.). EPPAT administration alone does not prevent metamorphosis or lead to the production of larval-pupal intermediates. This failure presumably reflects the concurrent suppression of JH production by the CA and the clearance of residual JH by alternative routes.

Thus, the present experiments document that the disappearance of JH necessary for the onset of metamorphosis normally occurs through two mechanisms: the inactivation of JH secretion by the CA and the production of JHE. Each mechanism can apparently alone clear sufficient JH to permit metamorphosis. Together they establish a potent system for the timely and thorough elimination of JH during the final days of larval life.

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Literature Cited

Bhaskaran, G., and G. Jones. 1980. Neuroendocrine regulation of corpus allatum activity in *Manduca sexta*: the endocrine basis for starvation-induced supernumerary larval moult. *J. Insect Physiol.* **26:** 431–440.

Bhaskaran, G., G. Jones, and D. Jones. 1980. Neuroendocrine regulation of corpus allatum activity in *Manduca sexta*: sequential neurohormonal and nervous inhibition in the last-instar larva. *Proc. Natl. Acad. Sci. USA* **77:** 4407–4411.

Bhaskaran, G. 1981. Regulation of corpus allatum activity in last instar *Manduca sexta* larvae. Pp. 53–82 in *Current Topics in Insect Endocrinology and Nutrition*, G. Bhaskaran, S. Friedman, and J. G. Rodriguez, eds. Plenum Publishing, New York.

Carrow, G. M., R. L. Calabrese, and C. M. Williams. 1984. Architecture and physiology of insect cerebral neurosecretory cells. *J. Neurosci.* **4:** 1034–1044.

Feyereisen, R. 1985. Regulation of juvenile hormone titer: synthesis. Pp. 391–429 in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 7, G. A. Kerkut and L. I. Gilbert, eds. Pergamon Press, New York.

Hammock, B. D. 1985. Regulation of juvenile hormone titer: degradation. Pp. 431–472 in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 7, G. A. Kerkut and L. I. Gilbert, eds. Pergamon Press, New York.

Nijhout, H. F. 1975. Axonal pathways in the brain-retrocerebral complex of *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Int. J. Insect Morphol. Embryol.* **4:** 529–538.

Safranek, L., and L. M. Riddiford. 1975. The biology of the black larval mutant of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* **21:** 1931–1938.

Safranek, L., and C. M. Williams. 1980. Studies of the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* **158:** 141–153.

Safranek, L., and C. M. Williams. 1987. Studies of the ecdysiotropic activity of juvenile hormone in pupae of the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* **172:** 299–306.

Sparks, T. C., B. D. Hammock, and L. M. Riddiford. 1983. The haemolymph juvenile hormone esterase of *Manduca sexta* (L.)—inhibition and regulation. *Insect Biochem.* **13:** 529–541.

Truman, J. T., L. M. Riddiford, and L. Safranek. 1973. Hormonal control of cuticle coloration in the tobacco hornworm, *Manduca sexta*: basis of an ultrasensitive bioassay for juvenile hormone. *J. Insect Physiol.* **19:** 195–203.

Truman, J. T., L. M. Riddiford, and L. Safranek. 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **39:** 247–262.

Vince, R. K., and L. I. Gilbert. 1977. Juvenile hormone esterase activity in precisely timed last instar larvae and pharate pupae of *Manduca sexta*. *Insect Biochem.* **7:** 115–120.